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Published in:
Fungal Biology

DOI:
10.1016/j.funbio.2015.04.005

Publication date:
2015

Citation for published version (APA):
Dagar, S. S., Kumar, S., Griffith, G. W., Edwards, J. E., Callaghan, T. M., Singh, R., Nagpal, A. K., & Puniya, A. K. (2015). A new anaerobic fungus (Oontomyces anksri gen. nov., sp. nov.) from the digestive tract of the Indian camel (Camelus dromedarius). *Fungal Biology*, *119*(8), 731-737. https://doi.org/10.1016/j.funbio.2015.04.005

Accepted Manuscript

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PII: S1878-6146(15)00059-8

DOI: 10.1016/j.funbio.2015.04.005

Reference: FUNBIO 575

To appear in: *Fungal Biology*

Received Date: 31 October 2014

Revised Date: 13 April 2015

Accepted Date: 20 April 2015

Please cite this article as: Dagar, S.S, Kumar, S., Griffith, G.W, Edwards, J.E, Callaghan, T.M, Singh, R., Nagpal, A.K, Puniya, A.K, A new anaerobic fungus (*Oontomyces anksri* gen. nov., sp. nov.) from the digestive tract of the Indian camel (*Camelus dromedarius*), *Fungal Biology* (2015), doi: 10.1016/ j.funbio.2015.04.005.

A new anaerobic fungus (*Oontomyces anksri* gen. nov., sp. nov.) from the digestive tract of the Indian camel (*Camelus dromedarius*).

Dagar, Sumit S[1*], Sanjay Kumar[1‡], Gareth W Griffith[2], Joan E Edwards[2], Tony M Callaghan[2], Rameshwar Singh[1#], Ashok K Nagpal[3] and Anil K Puniya[1†]

[1]Dairy Microbiology Division, ICAR-National Dairy Research Institute, Karnal - 132001, INDIA

[2]Institute of Biological, Environmental and Rural Sciences, Cledwyn Building, Aberystwyth University, Aberystwyth SY23 3DD, WALES UK

[3] ICAR-National Research Centre on Camel, Bikaner - 334001, INDIA

Present address:

[*] Bioenergy Group, Agharkar Research Institute, Pune – 411004, INDIA

[‡] College of Veterinary Medicine, University of Pennsylvania, USA

[#] Directorate of Knowledge Management in Agriculture, Krishi Anusandhan Bhawan-I, Pusa, New Delhi 110012, INDIA

[†]Corresponding author: Tel: +91-184-2259176; E-mail: akpuniya@gmail.com, Anil.Puniya@icar.org.in

Email addresses of other authors:- SS Dagar (dagarsumit@gmail.com), S Kumar (sanjayvrm@gmail.com), GW Griffith (gwg@aber.ac.uk), JE Edwards (joanee2002@hotmail.com), TM Callaghan (toneocallaghan@hotmail.co.uk), R Singh (rsndri@gmail.com), AK Nagpal (aknagpal@scientist.com), and AK Puniya (Anil.Puniya@icar.org.in)

Short Title: *Oontomyces anksri* gen. nov., sp. nov. from camel

ABSTRACT

Two cultures of anaerobic fungi were isolated from the forestomach of an Indian camel (*Camelus dromedarius* L.). Phylogenetic analysis using both the internal transcribed spacer (ITS) and large-subunit (LSU) regions of the rRNA locus demonstrated that these isolates were identical and formed a distinct clade within the anaerobic fungi (phylum Neocallimastigomycota). Morphological examination showed that these fungi formed monocentric thalli with filamentous rhizoids and uniflagellate zoospores, broadly similar to members of the genus *Piromyces*. However, distinctive morphological features were observed, notably the pinching of the cytoplasm in the sporangiophore and the formation of intercalary rhizoidal swellings. Since genetic analyses demonstrated this fungus was only distantly related to *Piromyces* spp. and closer to the polycentric *Anaeromyces* clade, we have assigned it to a new genus and species *Oontomyces anksri* gen. nov., sp. nov. Interrogation of the GenBank database identified several closely related ITS sequences, which were all environmental sequences obtained from camels, raising the possibility that this fungus may be specific to camelids.

Key words: Neocallimastigomycota; Indian camel; *Camelus dromedarius*; fungal taxonomy; rumen fungi; host specificity; *Oontomyces anksri*

Selected classifications: Anaerobic fungi; Host specialization; Rumen fungi; Symbiosis; Systematics

1. INTRODUCTION

Members of the phylum Neocallimastigomycota are a remarkable group of obligately anaerobic fungi, which normally reside within the digestive tract of mammalian herbivores. These fungi are important to the nutrition of their host, due to their significant role in the degradation of ingested lignocellulosic plant material, which the host itself is incapable of

utilizing. The potent fibre-degrading enzymes of anaerobic fungi, in addition to their physical disruption of the plant material, has led to recognition of their significant biotechnological potential, for example in biofuel processing and biogas production (Gruninger et al. 2014; Sirohi et al. 2013; Youssef et al.).

Since their belated recognition as Fungi (Orpin 1974), some 20 species have been reported (Griffith et al. 2009; Sirohi et al. 2012) but the taxonomic status of some of these species is uncertain (Eckart et al. 2010; Hibbett et al. 2007; Ho and Barr 1995; Ozkose et al. 2001). Following revision of the broader taxonomy of kingdom Fungi, this group is now considered as phylum Neocallimastigomycota, containing a single family, Neocallimastigaceae (in the order Neocallimastigales) (Hibbett et al. 2007). However, the status of the anaerobic fungi as a distinct phylum remains a matter of contention (Frey 2012; Powell and Letcher 2014).

The six genera within Neocallimastigomycota are divided into two groups based on their growth patterns: monocentric (*Neocallimastix, Piromyces* and *Caecomyces*) or polycentric (*Orpinomyces*, *Anaeromyces* and *Cyllamyces*), with the former growing as determinate thalli with a single sporangium and the latter forming more complex thalli with multiple sporangia (Griffith et al. 2009; Ho and Barr 1995). Two genera (*Neocallimastix* and *Orpinomyces*) form zoospores with multiple (7-30) flagella, in contrast to the uniflagellate zoospores of all other zoosporic fungi. Additionally, members of the genera *Caecomyces* and *Cyllamyces* are unusual since they form a bulbous holdfast rather than filamentous rhizoids. The advent of culture-independent methods for the study of these fungi has provided compelling evidence that additional genera of anaerobic fungi, as yet uncultured or unrecognized exist (Griffith et al. 2010; Kittelmann et al. 2012; Liggenstoffer et al. 2010; McGranaghan et al. 1999; Sirohi et al. 2013), and that some of these undescribed taxa may exhibit distinct host specificity (Liggenstoffer et al. 2010).

Here we present genetic and morphological data relating to a novel clade of anaerobic fungi isolated from the forestomach of the Indian camel (*Camelus dromedarius*), which is sufficiently distinct from the existing taxa of anaerobic fungi to merit its placement in a new genus *Oontomyces*.

2. MATERIALS AND METHODS

Liquor samples were collected using a stomach pipe from single-humped camel calf (Kutchchi breed male, 3 years-old, born domesticated), weighing 450 kg and maintained on a concentrate (50%) / roughage (50%) diet at the ICAR-National Research Centre for Camels (Bikaner, Thar Desert, Rajasthan, India; N28.001; E73.318; altitude 200 m). The strained liquor was brought to the laboratory in pre-warmed and O_2-free (gassed with CO_2) thermos flask. Isolations on cellobiose agar medium were performed at ICAR-NDRI, Karnal, as described by Dagar *et al.* (2011), including roll tube purification (Joblin 1981) to avoid the possibility of mixed cultures.

Taxonomic features were examined following growth on wheat straw medium for 3 days (Dagar *et al.* 2011) using phase contrast microscopy, and images were recorded using a Canon DS126191 digital camera. For genetic characterisation, the complete internal transcribed spacer (ITS; partial 18S, complete ITS 1, 5.8S, ITS 2 and partial 28S) and D1/D2 domain at the 5' end of the large-subunit (LSU) ribosomal DNA were amplified, using the primer pairs ITS1 (5′- TCC GTA GGT GAA CCT GCG G-3′)/ITS4 (5′- TCC TCC GCT TAT TGA TAT GC-3′) and NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′)/NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′), respectively (Dagar et al. 2011; Fliegerová et al. 2006). Care was taken to delimit the different regions of the rRNA locus in a consistent manner, as suggested by Hibbett et al. (1995), using the consensus sequences CATTA/CAACTTCAG

(end of 18S/start of 5.8S) and GAGTGTCATTA/TTGACCTCAAT (end of 5.8S/start of 28S). Phylogenetic reconstruction was conducted within the Geneious v6 bioinformatics package (Drummond et al. 2011), using MAFFT (v7.017 (Katoh et al. 2002)) for sequence alignment (default settings) and Mr Bayes for phylogenetic analysis (default settings; (Huelsenbeck and Ronquist 2001)).

3. RESULTS

After three days of growth from the original isolation tubes, two representative fungal colonies were selected and purified by repeated subculturing. Both the isolates (SSD-CIB1 and SSD-CIB2) formed uniflagellate zoospores (Fig. 1A, 1B) and filamentous rhizoids (Fig. 1C). Sporangia were formed terminally and varied in shape from ellipsoid to elongate (Figs. 1C-1E) (see http://www.forestphytophthoras.org/glossary/), as has been reported for several other species of anaerobic fungus (Dagar 2011; Gleason et al. 2002). However, sporangia were never mucronate (pointed), as is the case for the related *Anaeromyces mucronatus*. The sporangiophore (sporangium stalk) was often 2-3 times longer than the sporangium and separated from the rhizomycelium by a distinct constriction (Figs. 1D, 1E).
Intercalary rhizoidal swellings were frequently observed (Figs. 1F, 1G); these swelling bore some resemblance to the intercalary sporangia reported in *Orpinomyces intercalaris* (Dagar et al. 2011; Ho and Barr 1995) but none was ever seen to release or contain zoospores. Thus colony morphology was consistently monocentric (single sporangium per thallus) but confirmation using DAPI-staining and fluorescent microscopy that nuclei were restricted to sporangia (Ozkose et al. 2001) was not conducted.

Morphologically these new isolates conformed most closely to members of the genus *Piromyces*, in which nine species have been described (Ho and Barr 1995; Ho et al. 1993a, b; Kirk 2012). However, of these *Piromyces* species, none of the type specimens for these

species have been subject to both morphological and genetic analysis, except the rather distinctive *P. polycephalus* (recently renamed as *Anaeromyces polycephalus* (Chen et al. 2002; Kirk 2012)). Apart from *Piromyces cryptodigmaticus*, an uncultured organism defined by its ITS sequence alone (Kirk 2012), none of the type specimens or cultures are extant (Prof. Ho Yin Wan and Dr. Brigitte Gaillard-Martinie, pers. comms.). However, the pinching of the sporangiophore and highly variable sporangial shape (but not intercalary rhizoidal swellings) have been reported for *P. rhizinflata* (Breton et al. 1991).

Fig 1. Morphology of *Oontomyces anksri*

DNA sequences obtained for the ITS region (ca.700 bp amplicon; GenBank JX017310-11) of both isolates, and also for the D1/D2 domains of the LSU gene (ca. ≈780 bp amplicon; GenBank JX017314-15), were identical. More detailed analysis of the LSU region (Fig. 2; Suppdata 1) confirmed that these isolates were more closely related to *Anaeromyces* spp. than the *Piromyces* spp., which it resembled morphologically. Whilst *Anaeromyces* spp. also release uniflagellate zoospores, they form polycentric thalli with multiple sporangia.

Fig. 2. Bayesian backbone analysis of LSU sequences.

Suppdata. 1. ML analysis of LSU sequences.

Alignment of ITS sequences across the whole range of Neocallimastigomycota was unsatisfactory due to very presence of many gaps is such alignments. Therefore, analysis was restricted to only those genera forming uniflagellate zoospores (*Anaeromyces* / *Caecomyces* / *Cyllamyes* / *Piromyces*), and excluding the genera *Neocallimastix* and *Orpinomyces*, which formed a distinct clade in phylogenetic analysis of the LSU region (Fig. 3; Suppdata 2). The ITS sequences for Neocallimastigomycota lodged with GenBank

predominantly cover the ITS1 region, therefore, phylogenetic analysis was restricted to this region (bounded by the conserved sequences CATTA [3' end of 18S region] and CAACTT [5' end of 5.8S region), as suggested by Hibbett et al. (1995)). Following removal of duplicated sequences, and inclusion of closely related environmental nucleic acid sequences (ENAS), phylogenetic analysis was conducted on an alignment of 61 sequences (290 bp alignment). As with LSU analysis, the *Oontomyces* clade was recovered as a sister clade to *Anaeromyces* with high posterior probability support.

Fig. 3. Bayesian posterior probability analysis of ITS1 sequences.

Suppdata 2. ML analysis of ITS1 sequences.

4. DISCUSSION

The fact that the two isolates studied here form monocentric thalli and are thus clearly distinct from the polycentric genus *Anaeromyces* spp., as defined by Breton et al. (1990), indicates that the genus *Piromyces* (to which these fungi would have been consigned in the absence of genetic evidence) is polyphyletic, as previously suggested by Fliegerová et al. (2012). It is also apparent from Fig. 3 that several sequences lodged in GenBank and named *Anaeromyces* are also only distantly related to *Anaeromyces sensu stricto* (for which isolate JF1 [indicated in Figs. 2/3] is defined as the reference sequence [NCBI Reference Sequence: NR_111156.1] in the RefSeq Targeted Loci (RTL) database (Schoch et al. 2014). The most longstanding anomaly is *Anaeromyces* (formerly *Piromyces*) *polycephalus* (Chen et al. 2002), which is both morphologically and genetically distinctive, and in need of taxonomic reassessment, not least because it does not conform to the morphological circumscription of the genus *Anaeromyces*. For the isolates studied here, we propose below to assign these to a new genus, since they are similar in morphology to *Piromyces* spp. but genetically distant. Their monocentric thallus morphology prevents their assignment to the

genus *Anaeromyces*, as do several other morphological features. They are genetically distinct from *Anaeromyces sensu stricto*, being more closely related to *A. polycephalus* which they do not resemble morphologically.

Intriguingly, the most closely related ITS1 sequences to *O. anksri*, and which clearly fall within the *Oontomyces* clade, are part of a set of 155 ENAS sequences (JX944829-JX944983; Huo,X., Zhang,Z., Wang,N. and Zeng,J., unpublished). These sequences are all >89% identical across the ITS1 region, whereas the sister clades are <70% identical. These also originated from camel 'psuedorumen' (Bactrian camel; *Camelus bactrianus*) from Urumqi, Xinjiang, north-west China (N43.81; E87.58; altitude 830 m), some 2000 km north-east of Rajasthan.

The fact that this novel clade, which we formally name below, is very close to other sequences also isolated from camel raised the possibility that members of this clade exhibit host specificity. By far the most extensive culture independent study of anaerobic fungi is that of Liggenstoffer et al. (2010) (250,000 ITS1 GenBank sequences from a 454 NextGen sequencing project), in which the faeces of diverse (>30 species) herbivores from Oklahoma Zoo were studied. Several novel clades were discovered, some of which were apparently host-specific in equids. The absence of any sequences similar to *Oontomyces* from this dataset may relate to the fact that only one camelid host (*Lama glama*) was included, a finding that is consistent with the possibility of host specificity. Although the primers used by Liggenstoffer et al. (2010) are known not to be universal for all anaerobic fungi (Edwards et al. 2008), these primer sites are conserved in *Oontomyces* and thus would have amplified these sequences had they been present.

Camelids (family Camelidae; suborder Tylopoda) form a basal group within the class Certartiodactyla (which also includes whales, hippos, ruminants and pigs), with a distinctive gastrointestinal morphology, often described as pseudoruminant. The highly enlarged foregut comprises three distinct regions, analogous to the four chamber of true ruminants (suborder Ruminantia) and allows efficient digestion of plant lignocellulose via pre-gastric microbial fermentation (Van Soest 1994; Wilson 1989). This difference in foregut morphology is also associated with differences in protozoan populations, with several species (eg. *Entodinium ovumrajae* and *Calascolex camelinus*) found to be specific to camels (Dogiel 1947; Imai et al. 2004) and others that are common in true ruminants (e.g. cows, sheep) being absent (Kubesy and Dehority 2002).

Diagnosis

Oontomyces Dagar, Puniya & G.W. Griff. gen. nov.

Registration identifier: IF550795

Strictly anaerobic fungus with determinate, monocentric thallus with single terminal sporangium, and uniflagellate zoospores. The clade is defined by the sequences JX017310 (ITS1, 5.8S, ITS2 complete) and JX017314 (LSU, partial sequence). The most genetically similar genus is *Anaeromyces*, which is defined as forming a polycentric thallus (“Fungi semper anaerobici, tallus polycentricus, zoosporangia mucronata, zoospora uniflagellata”) (Breton et al. 1990), in contrast to the monocentric *Oontomyces*.

Registration identifier: IF550795.

Type species *Oontomyces anksri* Dagar, Puniya & G.W. Griff. sp. nov.

Etymology: “Oont” is from the Hindi, meaning “camel”.

Oontomyces anksri sp. nov. Dagar, Puniya & G.W. Griff. sp. nov.

Registration identifier: IF550796.

Holotype: SSD-CIB1 (ICAR-National Dairy Research Institute, Karnal, India)

Etymology: The specific name *anksri* is assigned in the honor of Dr. Anil Kumar Srivastava (Director, NDRI, Karnal) by taking the first two, one and three letters of his first, middle and surname (i.e. ANil Kumar SRIvastava = *ANKSRI*), respectively, who always encouraged us working in this under-explored area of microbiology.

Single terminal sporangium (70-100 µm long, 35-50 µm wide), ovoid to elongate, borne on a long sporangiophore (150-200 µm) which bears a distinct constriction delimiting the rhizoid from the sporangiophore. Ovoid to subovoid intercalary rhizoidal swelling are occasionally found (50-70 µm long, 40-60 µm wide). Zoospores are uniflagellate, spherical 5-7 µm in diameter, flagellum 24-30 µm in length (>3x longer than zoospore body). Obligate anaerobic fungus, isolated from camel forestomach. The structures originally examined are no longer extant nor are the pure cultures from which they were derived. The clade is defined by the sequences JX017310 (ITS1, 5.8S, ITS2 complete) and JX017314 (LSU, partial sequence). The type material for this species are the images contained in Figure 1 here and also a sample of freeze-dried forestomach fluid from which the cultures SSD-CIB1 and SSD-CIB2 were originally isolated; isotype material deposited at the Aberystwyth Fungarium, Wales (ABS) and Royal Botanic Gardens, Kew, UK (K).

5. ACKNOWLEDGEMENTS

Authors gratefully acknowledge Network projects on VTCC and NICRA for providing partial financial support during this study, and also for the award of a Stapledon Memorial Trust Travelling Fellowship (to SSD) and DBT-CREST Award Fellowships (to AKP), which permitted research visits to IBERS, Aberystwyth University. The authors are also grateful to SK Sirohi, RC Upadhyay, Parveen Malik, DN Kamra, NV Patil, RK Malik and SS Kundu for their support in conducting the work. TMC is grateful to the Aberystwyth University Postgraduate Studentship Scheme for funding. JE gratefully acknowledges funding from

BBSRC (Rumen Systems Biology; BBS/E/W/10964A01). We are also grateful to Dr. Paul Kirk (RBG Kew) for nomenclatural advice.

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FIGURE LEGENDS

Fig 1. Morphology of *Oontomyces anksri*. Zoospores (A, B) are uniflagellate, with the flagellum ca. 4 times the length of the spore body. Thalli are monocentric with sporangia normally being formed terminally (C-E). The shape of the sporangium was variable, ranging from elongate (C) to ovoid (D, E) and the sporangiophore usually (D, E) 2-3 times the length of the sporangium. A constriction is often visible at the base of the sporangiophore (arrowed, D, E). Intercalary rhizoidal swellings were also observed on some thalli (F, G). Figs. 1A, 1E are from isolate SSD-CIB1 and others from isolate SSD-CIB2. Scale bar indicates 10 µm (A,B) or 50 µm (C-G).

Fig. 2. Bayesian backbone analysis of LSU sequences (750 bp alignment of D1/D2 variable regions) of Neocallimastigomycota rooted with *Gromochytrium mamkaevae* (Chytridiomycota, order Gromochytriales). Bayesian posterior probabilities ≥ 0.75 are shown above the branches. The different genera of Neocallimastigomycota are shown in different coloured font with the *Oontomyces* clade in blue (and boxed). The reference sequence for *Anaeromyces* spp. is indicated by *. Scalebar indicates number of substitutions per site.

Fig. 3. Bayesian posterior probability analysis of ITS1 sequences of Neocallimastigomycota (290 bp alignment), including the genera with uniflagellate zoospores. The *Oontomyces anksri* clade is shown in blue font (*Anaeromyces* clade in red and the *P. polycephalus* clade in green. Line thickness is proportional to Bayesian posterior probabilities (thin lines = <0.7; thick lines >0.9) and PP probabilities are shown at salient nodes. * indicates the reference sequences for the genus *Anaeromyces*. Scalebar indicates substitutions per site and the tree is midpoint rooted.

Suppdata1. Maximum Likelihood tree of LSU sequences (750 bp alignment of D1/D2

variable regions; GTR substitution model) of Neocallimastigomycota rooted with *Gromochytrium mamkaevae* (Chytridiomycota, order Gromochytriales). Salient bootstrap values (as %; 1000 bootstrap replicates) are shown at nodes. Branches with >70% bootstrap support are drawn with thick lines. * indicates the reference sequence for the genus *Anaeromyces*. Scalebar indicates substitutions per site. The different genera of Neocallimastigomycota are shown in different coloured font with the *Oontomyces* clade in blue (and boxed). The reference sequence for *Anaeromyces* spp. is indicated by *. Scalebar indicates number of substitutions per site.

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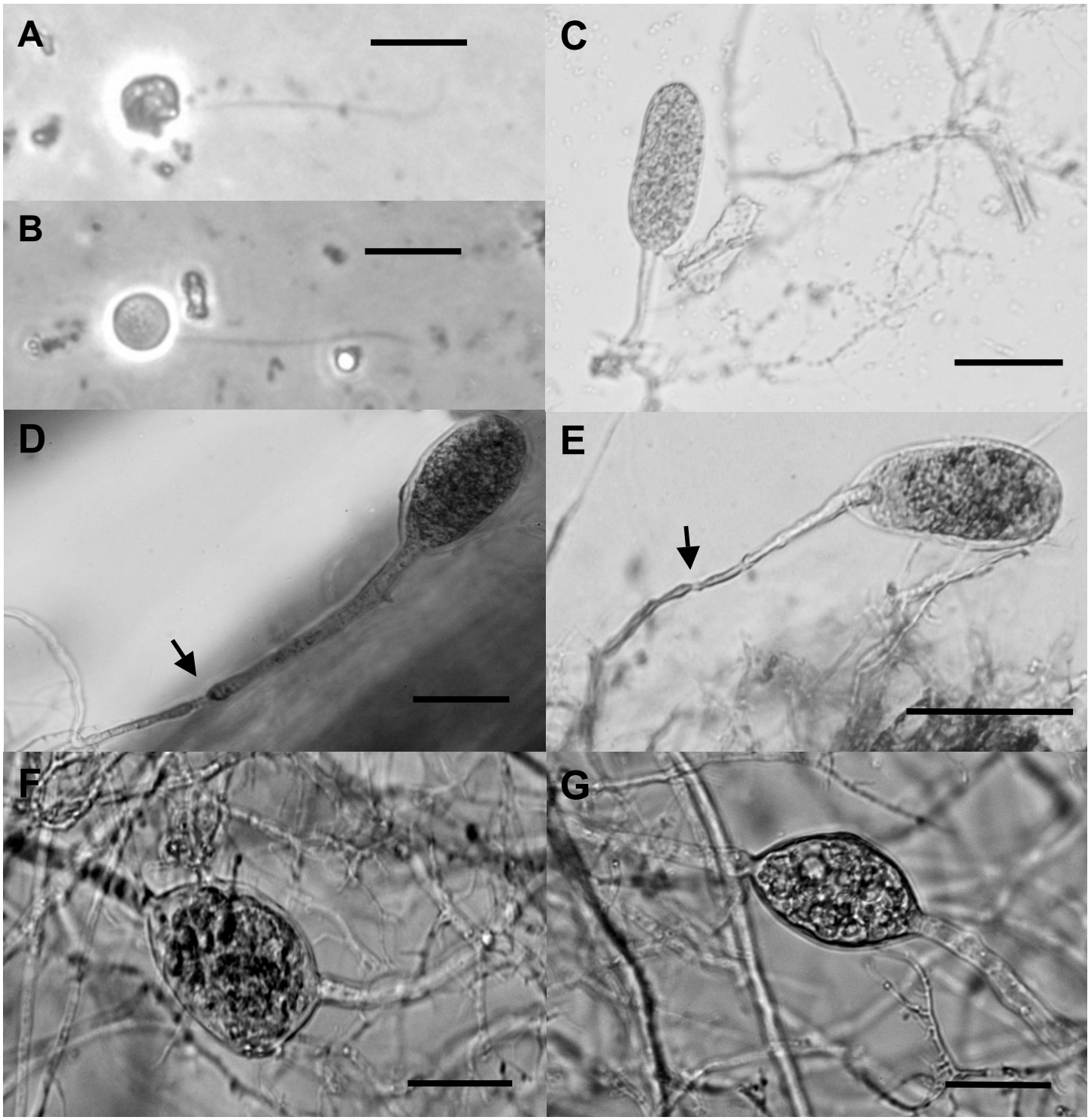

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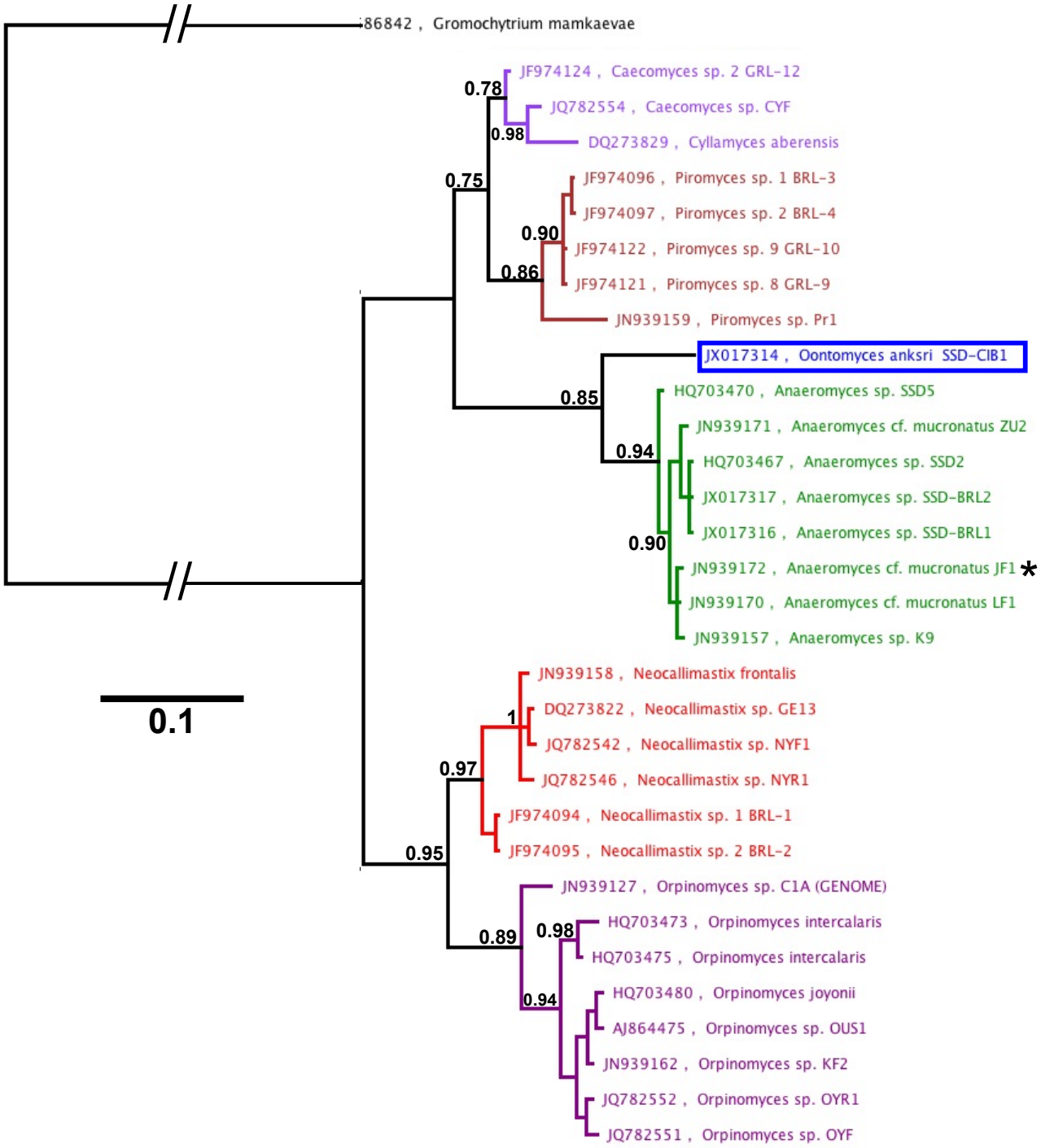

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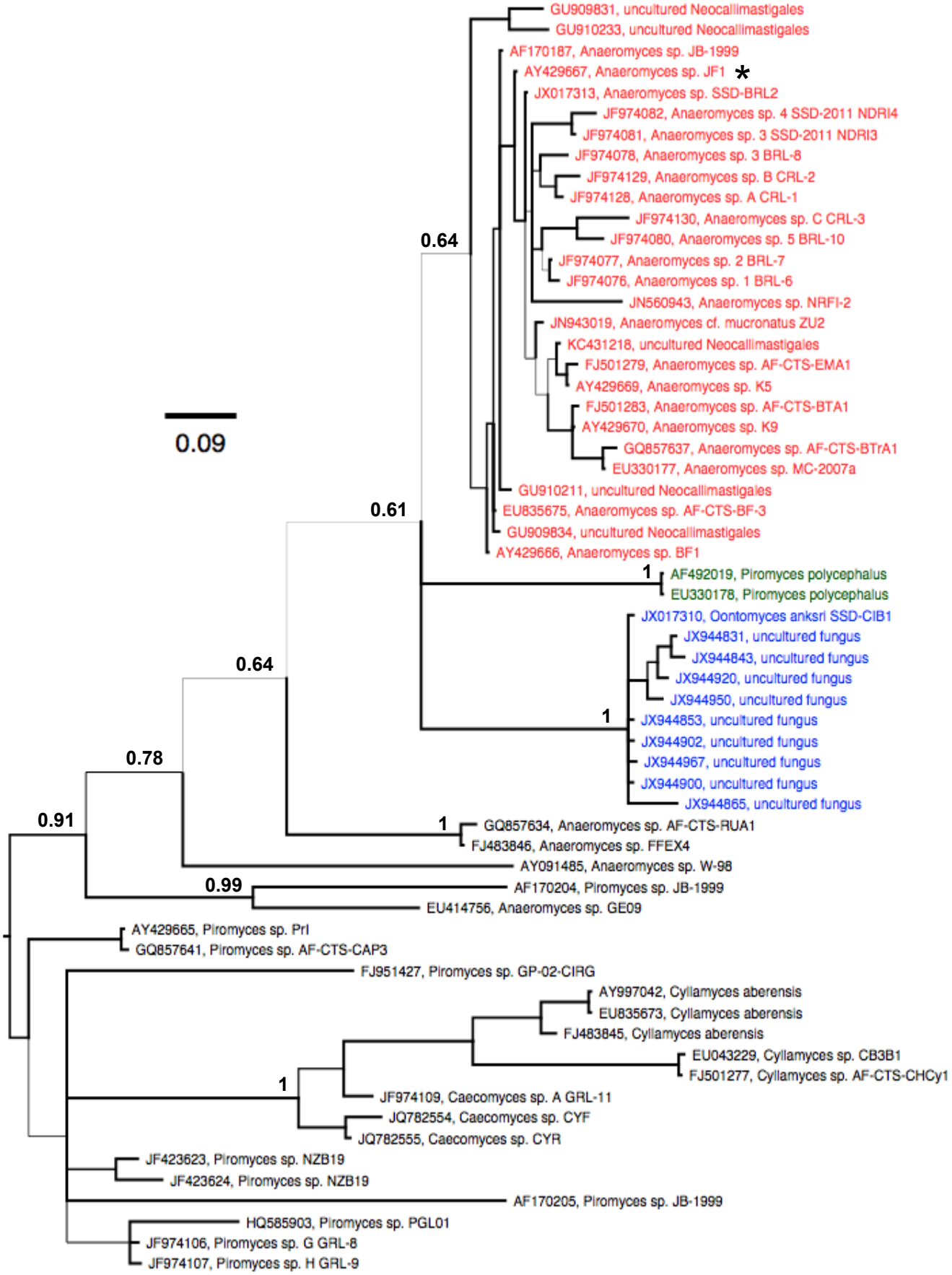

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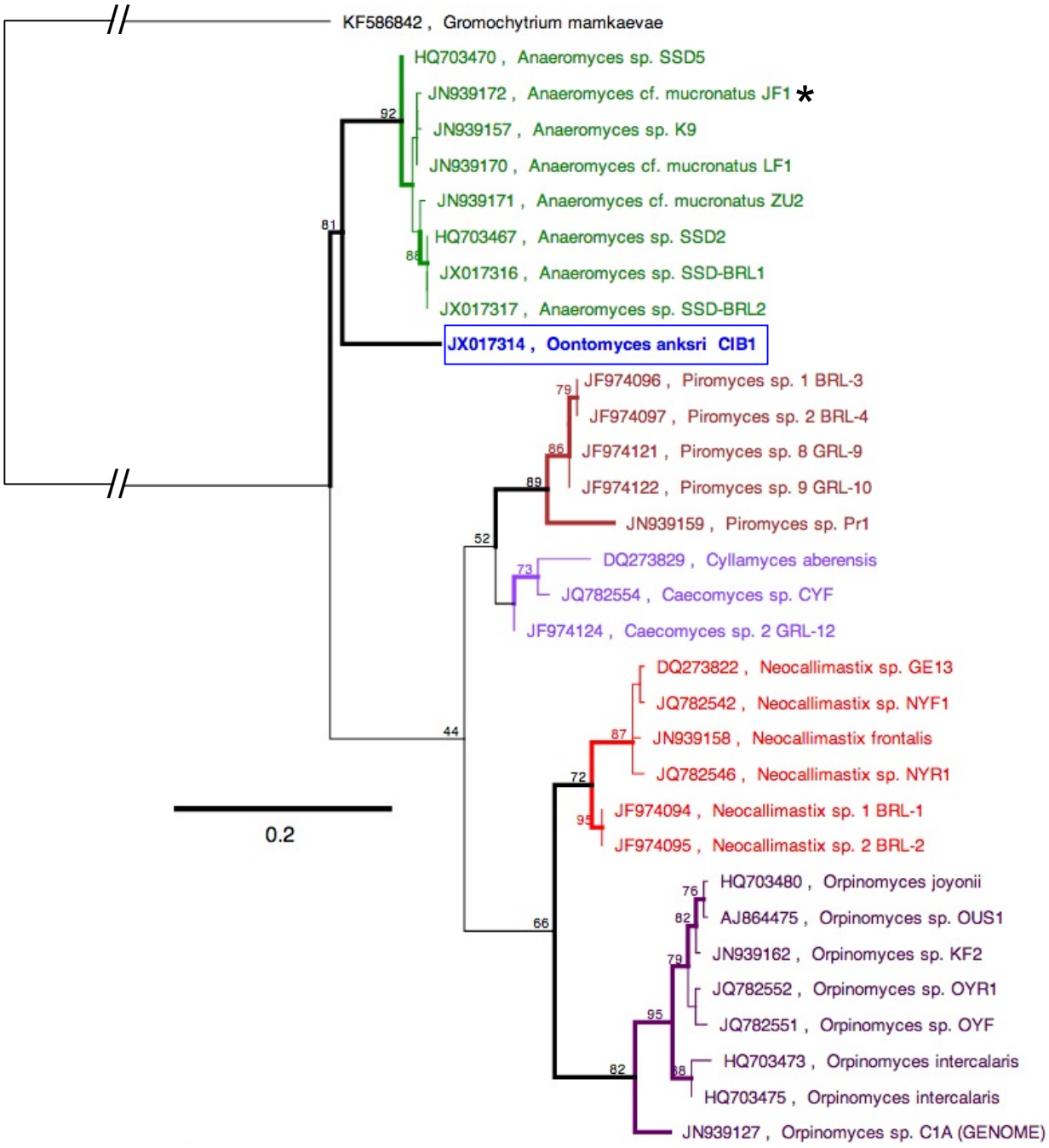

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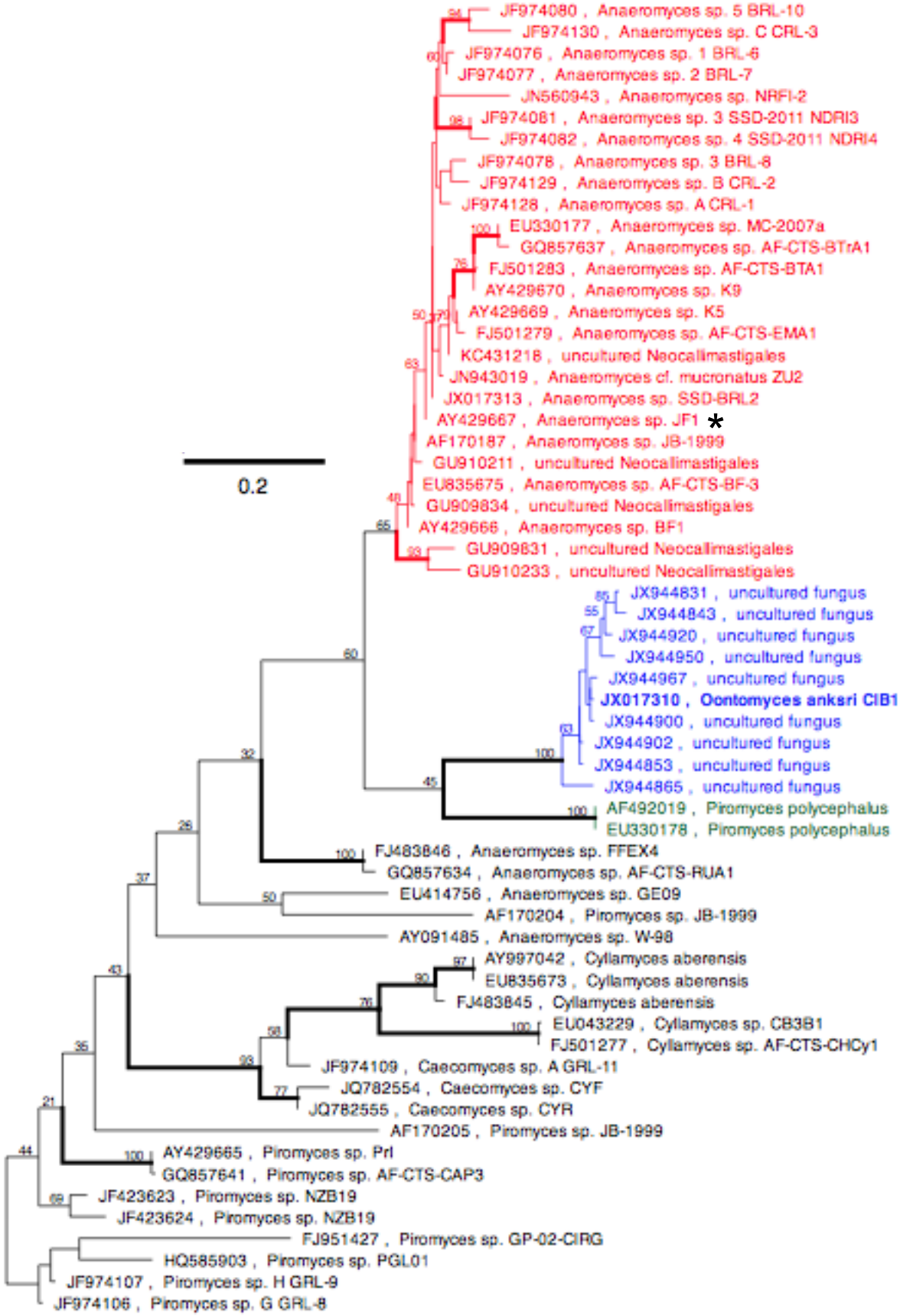

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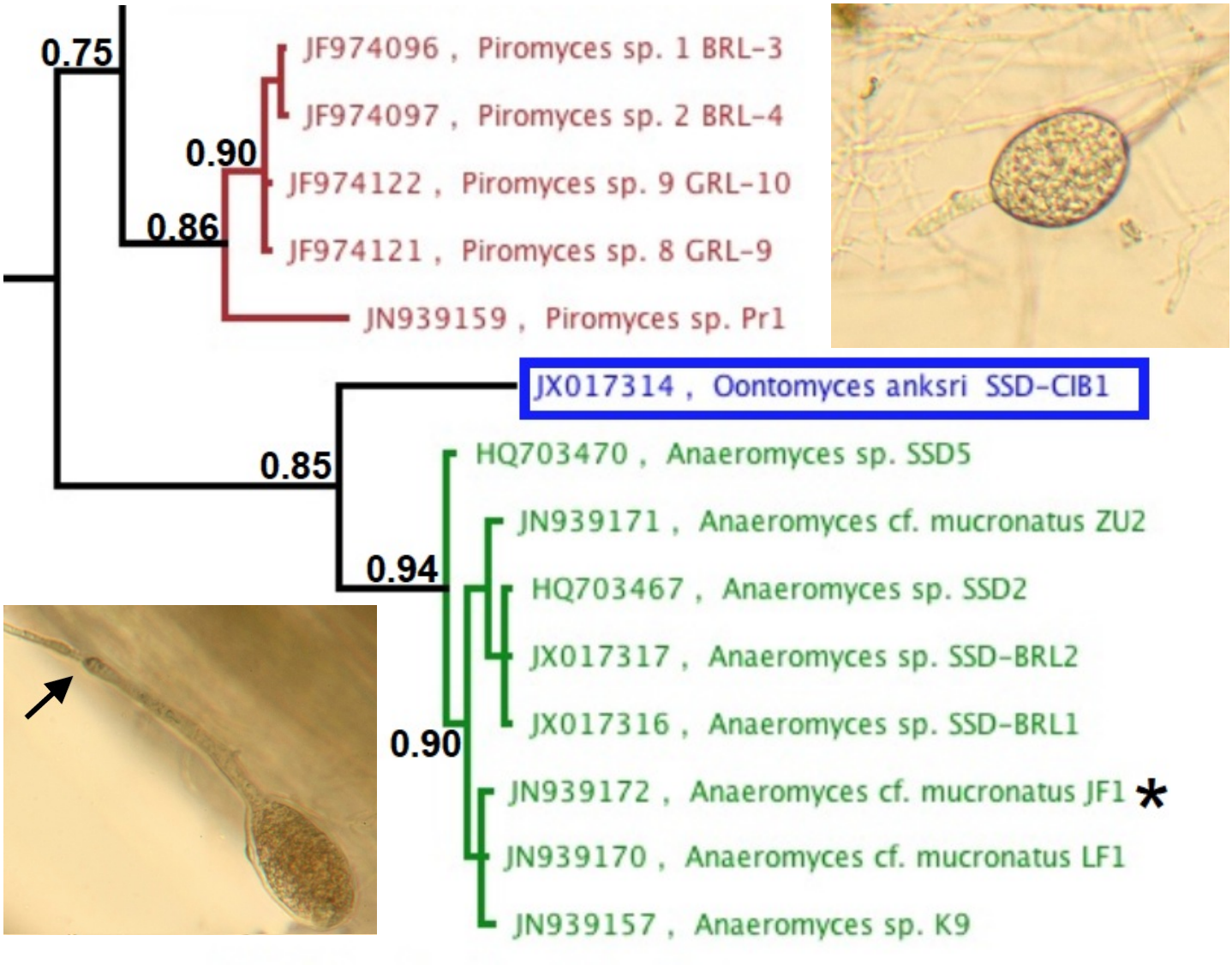
Graphical abstract



Research Highlights

- Two Neocallimastigomycota cultures were obtained from camel forestomach
- Cultures were monocentric and formed uniflagellate zoospores.
- ITS and LSU sequence analysis placed these in a distinct clade close to *Anaeromyces*
- Environmental sequences also from camel also fell into this clade
- This new fungus is formally named *Oontomyces anksri* gen. nov., sp. nov.